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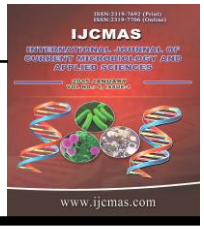

Original Research Article

Presence of Au-NPs in coelomic cells of earthworm *Eudichogaster prashadi* Stephenson

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Keywords

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A B S T R A C T

Earthworms harness the beneficial soil micro-flora to destroy pathogen, and converting organic waste into valuable products such as biofertilizers, biopesticides etc. Besides these, they have been considered to be valuable animals for interaction with inorganic materials and have been in constant touch with each other since inception of their origin. Due to this regular interaction, they sustained on this planet with well organized deposits of minerals. They can also produce nanoparticles either extracellular or intracellular routes. Present study revealed presence of gold nanoparticles (Au-NPs) in coelomic cells of earthworm *Eudichogaster prashadi*.

Introduction

Earthworms are free-living terrestrial animals living in soil, leaf litter under the stones and more heavily vegetated regions. Earthworms can 'biotransform' or 'biodgrade' chemical contaminants rendering them harmless in their bodies and can bioaccumulate in their tissues. They 'absorb' the dissolved chemicals through their moist 'body wall' due to interstitial water and also ingest by 'mouth' while passes through the gut. The coelomic fluid of their body contains free coelomocytes. Each segment of the body cavity is interfaced with the outer environment by dorsal pores enabling also the entering of microorganisms in coelomic cavity. Therefore, coelomic cavity of earthworms is not aseptic, it always contain bacteria, protozoa and fungi from the outer environment. Coelomic fluid of earthworms reportedly have anticoagulant, fibrinolytic, and antithrombotic activities (Hahn *et al*.,1997; Hrzenjak *et al*.,1998; Kim *et al*.,1998; Cooper *et al*.,2004;Lee *et al*.,2007; Trisina *et al*.,2011). Trisina and co-workers (2011) reported that the protein extracts from *Lumbricus rubellus* are responsible for antithrombotic and thrombolytic activities. In addition to proteins, glycosaminoglycans (chondroitin/dermatan sulfates and heparan sulfate) are also present in earthworm (*E. andrei*) extracts (Im *et al*.,2010) . Implications from leading researchers (Hillyer and Albrecht, 2001; Chitrani *et al*., 2006; Ulrich, 2006; Unrine *et al*.,2010)in earthworms revealed the recognition of nanoparticles involved in cellular uptake as

well as sub and intracellular events that further intriguing insights into earthworm's potentiality as biotransforming agent. As metal nanoparticles are being increasingly used in many sectors of the economy, there is growing interest in the biological and environmental safety of their production. The main methods for nanoparticle production are chemical and physical approaches that are often costly and potentially harmful to the environment. During the past decade, it has been demonstrated that many biological systems can transform inorganic metal ions into metal nanoparticles *via* the reductive capacities of the proteins and metabolites present in these organisms. The present study is focussed to characterize presence of gold nanoparticles in earthworm and to provide alternative platform for green synthesis of nanoparticles.

Materials and Methods

Collection of Earthworms

Eudichogaster prashadi (family Octochaetidae) were collected by digging and hand sorting method from different locations of Sagar MP, India during August - September, 2014. For identification, collected specimens were preserved in ethyl alcohol for molecular characterization, and also fixed in 4% formalin for morpho-anatomical study. Coelomic cells were extracted from live earthworms and sub-cultured in CO_2 incubator.

Isolation of Coelomocytes

Collected worms were thoroughly washed in running tap water before rinsing in distilled water and were not subjected to any control condition. Worms were placed on wet cotton to ensure complete defecation in order to avoid contamination during harvesting of coelomocytes. After 2-3 hrs, worms were wiped with cotton wool soaked with 70 % ethyl alcohol to avoid any further contamination. The surface cleaned worms were placed alternately in sterile petridish containing cold extrusion buffer (NaCl 71.2mM; Ethanol 5%; Guaicol-glycerol-ether 50.4mM; EGTA 5mM, pH 7.3) and distilled water at interval of one minute for 8-10 times. Coelomic fluid extruded out through dorsal pores due to external stress condition. After collection of coelomic fluid in cold extrusion buffer, worms were released in soil.

Culturing of Coelomocytes

The excreted coelomic fluid was pipette into tubes filled with LBSS solution (NaCl 71.5mM; KCl 4.8mM; $MgSo_4.7H_2O$ 1.1mM; KH_2PO_4 0.4mM, pH 7.3) and centrifuged at 4°C for 5 min. Loose pellets of coelomocytes were washed 2-3 times with cold LBSS solution. Cell count was maintained 10^7/ml with trypan blue exclusion. The isolated coelomocytes were loaded in petridish with DMEM supplemented with 10% FBS and incubated for 3 days in CO_2 incubator.

Characterization of Nanoparticles

The coelomic cells were observed in fluorescence and confocal microscope and later confirmed in transmission electron microscopy (TEM) after following routine techniques of microscopy.

Flow Cytometeric Assessment of Coelomic Cells

The coelomic cells were examined using flow cytometeric method (Kumar *et al*., 2011). 50µl sample and control (distilled water) was added into 950µl PBS and analyzed with flow cytometer (FACS Canto

II BD Biosciences, San Jose, CA) using FACS Diva 6.1.2 software (BD Biosciences). In the dot plots, X-axis reflects the FSC intensity in logarithmic scale, and Y-axis corresponds to the SSC intensity in linear scale. The gating of the data was based on SSC and FSC of the coelomic cells and control, respectively. This allowed us to differentiate the cells in which nanoparticles were present. Dead cell discrimination of the cells was carried out according to the protocol described by Jung *et al*. (2015) using propidium iodide dye.

Results and Discussion

Systematic Enumeration

Study site: Sagar (MP); Rajghat dam(latitude 23°43'51"N; longitude 78°45'22" E; elevation 393m); Silera (latitude 23°51'37"N; longitude 78°39'17"E; elevation 490m); Jaruakheda (latitude 23°56'16"4N'; longitude 78°31'77"E; elevation 486m); Dhana (latitude23°43'99" N; longitude 78°52'91" E; elevation 495m), Bilhera (latitude 23°39' 94" N; longitude 78°44'54" E; elevation 531m); Surkhi village (latitude23°37'34" N; longitude 78°50'25" E; elevation 505m), Gugwara (latitude 23°31'27"N; longitude 78°56'81" E, elevation 428m); Patkue village (latitude 23°52'57" N; longitude 78°47'27.144" E; elevation 352m), Baroda (latitude 23°54'81"N;longitude 79°07'35"E; elevation 477m), Gousra village(latitude 23°43'14" N; longitude 78°46'90" E; elevation 505m); Shahpur (latitude 23°53'40" N; longitude 79°02"71" E; elevation 400m); Dhoha (Latitude 24°00'69" N; longitude 78°38'78" E; elevation 432m), Rajbans (latitude24°09'48" N; longitude 78°35'83" E; elevation 445m); Ghureta village (latitude23°49'12" N; longitude 78°53'80"E, elevation 489m).

Diagnosis (Fig 1): Length 75-105 mm, diameter 5-9 mm, 140-152 segments;colour yellowish brown, with only a slight difference between dorsal and ventral surfaces;prostomiumprolobic. Dorsal pores from 11/12 or 12/13.In general setae *aa*=3.2-3.6; *ab*=1.3-1.6; *bc*= 2.5-2.6; *cd*=0.27-0.28; *dd*on xii. Male genital field tumescent without special demarcation, on xvii-xix, with or without a deep slit-like depression along xiii, and with a deep longitudinal depression on xvii and xix. Male pores minutes, in seminal grooves, on the setal arc of xviii, at *ab*; prostatic pores minute, at the ends of seminal grooves, on xvii and xix, at *ab*, seminal groove biconcave. Spermathecal pores minute, on the setal arcs of viii and ix at *ab*. Genital markings circular to oval. Septum 4/5 thin, 5/6-9/10 moderately strengthened, 10/11 slightly so, 11/12 still less so. Gizzards in v and vi large rounded and firm. Calciferous glands shortly stalked, in xi and xii. Intestine begins in xv. Last heart in xii. Nephridia in five longitudinal rows on each side of the body. Supra-intestinal glands 7-9 pairs, in 1xxvi-1xxxix. Holandric, testes and male funnels free, in x and xi; seminal vesicle in ix and xii, Each spermatheca with a flat, disc-like, multi-loculateental diverticulum, duct about as long as or slightly shorter than ampulla of spermathecae.

Habitat

Loamy soil, usually found in river bedside grasslands.

Biology

Hermaphrodite, copulation generally occurs in rainy season between August and September.

Molecular Characterization

Collected earthworms were identified with the help of available literature (Gates, 1972; Julka,1988), later re-confirmed with amplified 683 bp cytochrome oxidase *coi*-I gene. The universal primers, LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAG GGTGACCAAAAAATCA-3') were used to amplify *coi*-I gene sequences. Master mix used for PCR reactions contained 1 U Taq polymerase (JonakiTaq, CCMB, Hyderabad, India), 1.5mM $MgCl_2$, 0.2mM of each primer, 0.125mM of each deoxynucleotide. Thermal cycling was done in the ABI thermocycler with following conditions of PCR; 4 min initial denaturation at 94°C, 33 cycles of 1 min denaturation at 94°C, 1 min annealing at 45°C, 1 min elongation at 72°C, and a final elongation at 72°C for 10 min followed by 4°C for 10 min. The PCR products were visualized on 1.0 % agarose gels with 1 X TAE buffer and 0.5 µg/mL EtBr. The PCR products of the expected size were purified using the QIAquick Gel Purification Kit (QIAGEN GmbH, Hilden, Germany) following the manufacturers' protocols. Purified PCR products were sequenced using BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California) on an ABI3500 with LCO 1490 - HCO2198 primers (Xcelris Genomics Pvt. Ltd., Ahmedabad, India). The electropherograms were processed and analyzed with Bio-edit (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and phylogenetic analyses was conducted using MEGA v6.

Characterization of Nanoparticles

Uv-spectra of the particles shown the presence of gold nanoparticles as well large aggregates of nanoparticles were observed in the coelomic fluid in all microscopic observation (Fig 2-3). The nanoparticles were primarily with an average diameter of 20-30nm. The collections of discrete nanoparticles were randomly selected to measure average diameter (Fig 4). Clear fringes in the coelomic cells further confirmed the spherical structure of nanoparticles. The flow cytometery observation of coelomic cells demonstrated 24.60% intensity of the side scatter (SSC)) which was more pronounced with control (Fig5) confirms the presence of nanoparticles in coelomic cells. It has been known that earthworms are able to reduce metal ions in various organs and tissues remote from the portal of entry. The bioaccumulation study revealed that metals ate usually deposited in the form of nanoparticles. Certainly there exist certain limitations that should be taken into account for harvesting of nanoparticles from earthworms. As the size and shape of nanoparticles depends on their localization or habitat of the worm which may depend on differences in the content of metal ions in various tissues These factors could influence the level of metal deposition and also the prospect of new nucleation events *i.e.* initiation of nanoparticle formation. The hetero-aggregates of nanoparticles produced hinder the efficient extraction, isolation and purification of nanoparticles from their tissues.

Proposed Mechanism of Synthesis of Nanoparticles in Worms

On the whole, the mechanism of metal nanoparticle synthesis in their tissues may includes three main phases: *the activation phase,* metal ions reduction and nucleation of the reduced metal atoms; *the growth phase,* small adjacent nanoparticles spontaneously coalesce into particles of a larger size; *the termination phase,* determining the final shape of the

nanoparticles (Sharma *et al*., 2002; Raveendran *et al*., 2003; Govindaraju *et al*., 2008). If duration of the growth phase increases, nanoparticles aggregate to form variety of irregularly shaped nanoparticles viz., nanotubes, nanoprisms, nanohexahedrons etc. (Harris *et al*., 2008). However, at termination phase, nanoparticles acquire the most energetically favourable conformation, with this process being strongly influenced by the ability of ingested organic matter to stabilize metal nanoparticles. The reduction process of metal ions with the formation of nanoparticles is affected by a large number of factors besides the ecological niche of the worm. Various organic matters containing active biomolecules in different combinations and concentrations also effect the synthesis of nanoparticles in the coelomic fluid of the worms.

Fig.1 Diagnostic characters of *Eudichogaster prashadi* : a, dorsal view of ; b,genital region; c,spermathecae with sperm; d,testes with funnels; e,genital markings; e,location of gizzard; e,typhlosole

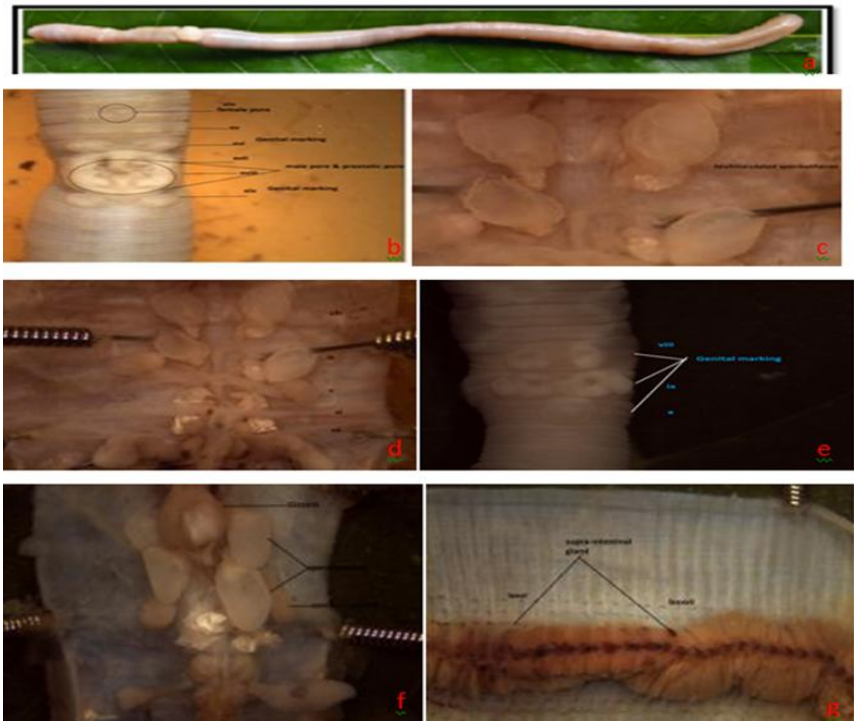


Fig.2 View of coelomic cells of *Eudichogaster prashadi* with nanoparticles in fluorescence microscope

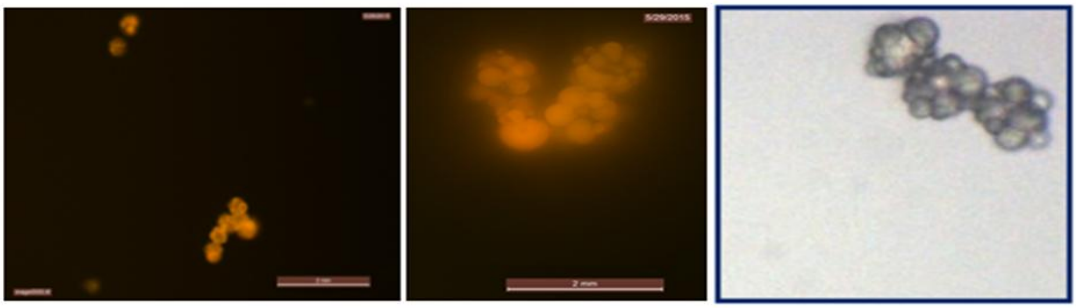


Fig.3 View of coelomic cells of *Eudichogaster prashadi* with nanoparticles in confocal microscope

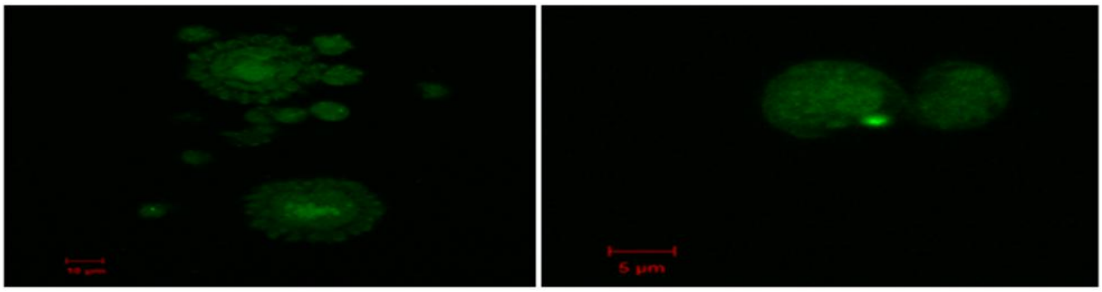


Fig.4 View of coelomic cells of *Eudichogaster prashadi*:a, nanoparticles in coelomic cells; b, isolated nanoparticles from coelomic fluid; c, aggregates of nanoparticles in coelomic fluid; d, nanoparticles segregates extracted from coelomic fluid.

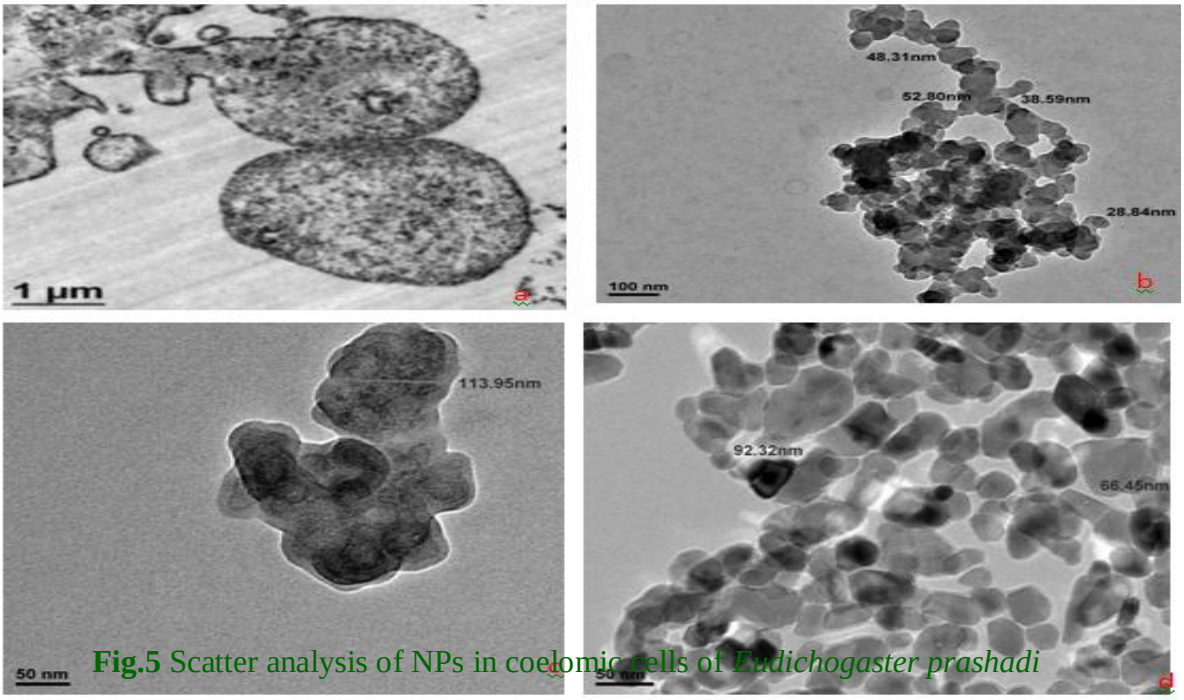
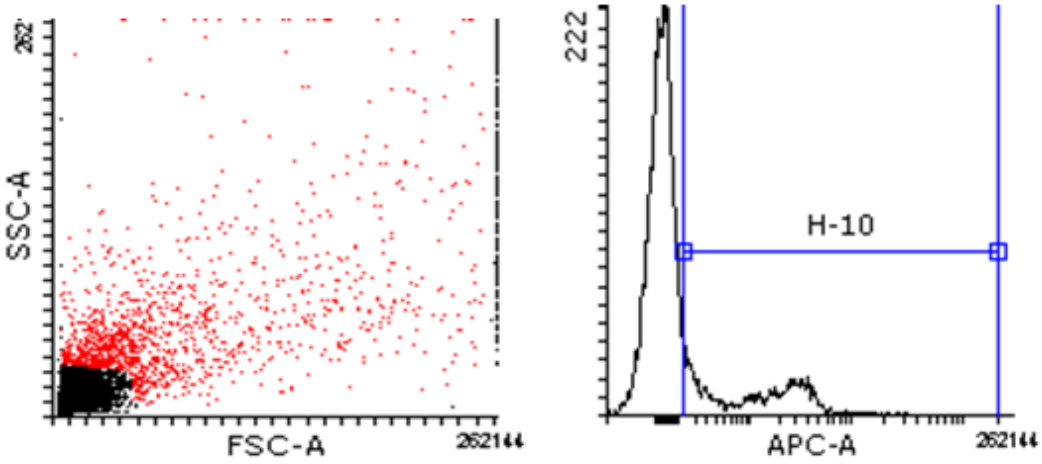


Fig.5 Scatter analysis of NPs in coelomic cells of *Eudichogaster prashadi*



Interaction of Nanoparticles and Coelomic Cells

The NPs can also be excreted by nephridia, while agglomerated /hetero aggregated NPs can be eliminated by process of encapsulation. The internalization of NPs in coelomic cells occurs due to TLR (toll like receptors) and their recognition is mediated by pattern-recognition receptors (PRRs, which leads into various inflammatory cytokines and antimicrobial peptides. Coelomic cytolytic factor (CCF) is a well-characterized 42-k Da lytic protein (secreted into coelomic fluid in a stable form) in earthworms that act as pattern-recognition molecule and is present on the cells of the mesenchymal lining of coelomic cavity as well as on free coelomocytes. CCF is formed by two spatially distinct lectin like domain located in the central part of the molecule which interacts with lipo-polysaccharide and β-1,3,glucans and second domain is located in C-terminal part interacts with peptidoglycan constituents. Upon binding of PAMPs, CCF triggers the activity of prophenoloxidase (ProPO) cascade. The ProPO cascade is sensitive and efficient defense system consisting of several proteins such as zymogenic proteinases, proteinase inhibitors, ProPO, PO, PRRs with final product melanin. Melanin exhibits fungi-state, bacterio-state and antiviral properties and is involved in wound healing & defense reaction. This melanization reaction accompanies the cellular defense reaction encapsulation, resulting in the formation of so-called brown bodies of NPs.

Coelomic cells, eleocytes play an important role in immune responses producing bacterial substances (Valembois *et al*., 1982; Ville *et al*., 1995; Milochau *et al*., 1997) and also participates in reaction of encapsulation and formation of brown bodies (Cooper and Stein,1981). The number and composition of coelomic cells depends on exogenous (environmental) as well as endogenous (biotic, life cycle) factors. Parry (1975) proved short-term and limited memory in coelomic cells of earthworms in transplantation experiments to autografts and xenografts. Van der Ploeg *et al*., (2013) reported enzymes involved in antioxidant mechanism doesn't effect on exposure of C_{60} fullerenes on *L. rubellus*. However, coelomic cytolytic factor 1(CCF1), a pattern recognition receptor was found suppressed in lifelong experiment. Clathrin-mediated endocytosis, caveolae-mediated phagocytosis and micro-pinocytosis may involve in uptake of NPs by coelomocytes. Scavenger receptor class A (a pattern recognition receptors) of coelomocytes is potential pathway to phagocytosis the NPs by amebocytes of earthworms. Recently scientists become more and more interested in the interaction between inorganic molecules and biological species. Studies have found that many microorganisms can produce inorganic nanoparticles through either intracellular or extracellular routes. During cellular immune responses coelomocytes play an important role in phagocytosis, inflammatory processes, graft rejection and coagulation of coelomic fluid. During the humoral immune response, they secrete lysozyme, agglutinin, peroxidase, phenoloxidases and antimicrobial factor (fetidin, lysenin, eiseniapore, coelomic cytolytic factor). These cytotoxic molecules increase the intracellular calcium concentration that participates in exocytosis.

In conclusion, nanoparticles produced by a biological process are always far superior, in several ways, to those particles produced by chemical methods. Despite that the chemical methods are able to produce large quantities of nanoparticles with a defined size and shape in a relatively short time. However,

they produce hazardous toxic wastes that are harmful, not only to the environment but also to human health. With biological process, the use of expensive chemicals is eliminated, and the more acceptable green route is not as energy intensive as the chemical method and is also environment friendly. Present study revealed the green synthesis of gold nanoparticles in coelomic fluid of particular species of earthworm *Eudichogsater prashadi* may have potential for industrial application. However the mechanism of synthesis of these particles and their presence in different ecologically important species need to be further investigated.

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